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# Prospects for Hybrid Breeding in Bioenergy Grasses

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## Disciplines

Agricultural Science | Agronomy and Crop Sciences | Plant Breeding and Genetics

## Comments

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## Prospects for hybrid breeding in bioenergy grasses

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### Abstract

Biofuels obtained from biomass have the potential to replace a substantial fraction of petroleum-based hydrocarbons that contribute to carbon emissions and are limited in supply. With the ultimate goal to maximize biomass yield for biofuel production, this review aims to evaluate prospects of different hybrid breeding schemes to optimally exploit heterosis for biomass yield in perennial ryegrass (*Lolium perenne* L.) and switchgrass (*Panicum virgatum*), two perennial model grass species for bioenergy production. Starting with a careful evaluation of current population and synthetic breeding methods, we address crucial topics to implement hybrid breeding, such as the availability and development of heterotic groups, as well as biological mechanisms for hybridization control such as self-incompatibility (SI) and male sterility (MS). Finally, we present potential hybrid breeding schemes based on SI and MS for the two bioenergy grass species, and discuss how molecular tools and synteny can be used to transfer relevant information for genes controlling these biological mechanisms across grass species.

## Introduction

Biofuels have the potential to replace a substantial fraction of petroleum-based hydrocarbons that contribute to carbon emissions and are limited in supply. Maize, sugarcane, and soybean are currently used to produce biofuels [1]. However, additional crops that are not directly compromising feed and food production are required to implement more sustainable bioenergy production systems. As part of the efforts to tap renewable energy sources, the Department of Energy (DOE) created the Bioenergy Feedstock Development Program (BFDP) to identify and evaluate plant species with promising biofuel potential [2-5]. The screening for energy crops included 100 species of short-rotation trees and 35 herbaceous species [6], and identified four woody species and switchgrass (*Panicum virgatum*) as model species for bioenergy research [7]. Grasses, especially perennial grasses grown for forage production, are advantageous for bioenergy production because due to their perennial nature, farming practices such as tillage are not required as often as for annual crops [8]. Moreover, they have low water and nutrient requirements, a high rate of carbon fixation, and are consequently effective in biomass production [9]. In addition, perennial grasses have a low lignin/cellulose ratio, convenient for biomass conversion.

Both of our target species are characterized by a highly effective self-incompatibility (SI) system which promotes cross pollination, thereby maintaining a high level of heterozygosity [10]. Such allogamous species are usually improved as population or synthetic varieties, which exploit heterosis only partially. To increase productivity and sustainability of grasslands for bioenergy production, novel tools and innovative breeding strategies are needed. Hybrid varieties have the potential to outperform populations and synthetic cultivars. Indeed, heterosis for traits related to biomass yield such as plant height and dry matter yield have been described for both, perennial

ryegrass and switchgrass hybrids. For switchgrass, hybrids have been assessed for yield on single plant and sward level in field trials: in a spaced plant study, the hybrids of selected genotypes from the cultivars 'Kanlow' and 'Summer' yielded between 0.18 (15.4%) and 0.35 (30%) kg per plant more biomass than the average of both parents [11]. When the corresponding cultivars were evaluated in swards, high parent heterosis ranged from 30 to 38% [12]. Similar studies to determine heterosis for dry matter yield (ADMY) in swards were performed for ryegrass [13-15]. The genetically most distant crosses yielded 15.5 t/ha ADMY, which represented panmictic mid-parent heterosis of 13% [13]. For spaced plants, heterosis was as high as 31% in population hybrids of perennial ryegrass [16,17].

Self-incompatibility (SI) and male sterility (MS) are important biological mechanisms which can be exploited to control pollination in hybrid breeding schemes. Despite intense research efforts during the last decades, little is known about the genetic control of SI and MS in perennial grasses such as ryegrass and switchgrass. For the gametophytic SI system which is assumed to be conserved in grass species [18], molecular genetic characterization is most advanced in perennial ryegrass [19], enabling the opportunity to use ryegrass as a model species for the genetically more complex switchgrass. Similarly, cytoplasmic MS has been characterized and commercially used in rye [20] and other annual grass species such as maize [21].

Conserved synteny between grass genomes provide the opportunity to transfer genetic knowledge and sequence resources between grass species [22]. Approximately 30 large chromosomal blocks are present in most grass genomes with different rearrangements [23-27]. The level of synteny among grass genomes of species such as rice, barley, maize, sorghum, Brachypodium and others is between 40 and 73% [28]. More recent studies proposed that grass genomes have evolved from a common ancestor genome with a minimal size of 33.6 Mb

structured in five proto-chromosomes [29] from which grass species evolved by whole genome or segmental duplications, diploidization, small-scale rearrangements (translocations, gene conversions), and gene copying events [30]. Microsynteny can be used for identification of orthologous genes or genome regions in related species. Information available on complete and ongoing grass genome sequencing projects such as for rice, maize, sorghum, Brachypodium and possibly soon for bioenergy grass species such as ryegrass, switchgrass and *Miscanthus sinensis* will benefit marker development and gene identification in non-model bioenergy grass species [23,31].

This review will focus on two perennial grass species for bioenergy production, namely switchgrass (*Panicum virgatum*) and perennial ryegrass (*Lolium perenne* L.). As perennial ryegrass is one of the few diploid perennial grass species for which substantial genomic tools are available [32,33], the potential to use synteny for the transfer of genetic knowledge and sequence resources between grass species is elucidated. In particular, this review aims to 1) evaluate current population and synthetic breeding schemes, 2) to review heterotic patterns that suggest possibilities to exploit heterosis, and 3) to address limitations and opportunities provided by the reproductive biology of these grasses

### **Current population and synthetic breeding schemes in switchgrass and perennial ryegrass**

Ryegrass and switchgrass are mainly developed as population or synthetic cultivars due to SI systems, which promote outcrossing in both species [34-36]. Whereas population breeding is characterized by a continuous improvement through recurrent selection, synthetic breeding refers to crosses among a limited number of selected parents followed by multiplication through repeated open pollination. Both, population and synthetic cultivars, represent panmictic populations since they are based on open pollination ensuring random mating (Posselt, 2010).

These population-based breeding schemes have been continuously improved. For example, Casler and Brummer (2008) determined by simulation studies that among and within family selection yielded higher genetic gains compared to recurrent selection for half sib and full sib families in most cases. For yield related traits, population improvement based on phenotypic or genotypic selection has been applied for several years resulting in low genetic gains. Humphreys (1997) reported an average yield gain of 4% per decade for forage yield which is approximately four times lower than the gains achieved for grain crops. This limited progress is likely due to negative correlations between the different target traits forage yield, seed yield, and forage quality [37,38] and long breeding cycles in allogamous perennial grass species [39]. Another reason might be incomplete exploitation of heterosis in population and synthetic breeding schemes [37].

On the positive side, open pollinated cultivars are heterozygous and heterogeneous. Heterozygosity can result in stable yields as reported for sorghum [40,41]. Other studies in faba bean (*Vicia faba*) [42] and barley [43] have demonstrated that genetic diversity within a population can provide a buffer in response to biotic or abiotic stress. For yield improvement, narrow based synthetics might be advantageous due to higher selection intensity compared to broad based synthetics or traditional population varieties obtained by recurrent selection schemes. However, narrow based synthetics are susceptible to inbreeding depression [44]. Therefore, the optimal number of founders in a synthetic cultivar might be a trade-off between the genetic diversity available and the selection intensity required for efficient polycross breeding. Choosing a diverse set of parents aided by molecular markers is a possible strategy to reduce the impact of inbreeding depression, in particular with low-cost markers becoming increasingly available [44]. In perennial ryegrass, progeny from wide crosses are consistently

higher yielding. Molecular markers can be efficiently used to assess diversity among parental genotypes to determine the optimum number of genotypes of a polycross [45]. A major advantage of population or synthetic compared to hybrid varieties is, that seed production is possible at substantially lower costs. Thus, from a farmer's perspective, lower yields might be compensated for by lower seed costs.

### **Prospects for hybrid breeding in perennial grasses**

Hybrid performance is mainly determined by the degree of heterosis, which is defined as superiority of the heterozygous hybrid over its homozygous parents [46]. Heterosis displayed by F<sub>1</sub> progeny of two random mating populations is known as panmictic-midparent heterosis [44]. In allogamous grass species, the contribution of heterosis to hybrid performance is difficult to estimate due to heterozygosity of parents and confounding polyploidy effects [47-49]. Biologically, there is a potential to more efficiently exploit heterosis in hybrid breeding schemes in perennial bioenergy grasses, because these cross-pollinated species are (i) wind pollinated, (ii) produce a large amount of seed per plant, and (iii) exhibit a strong self-incompatibility mechanism which can be used for cost-effective hybrid seed production.

### ***Identification of heterotic patterns and development of heterotic groups***

An important requirement to efficiently exploit heterosis in plant breeding is the identification or development of heterotic groups as available in maize [46]. Heterotic groups are complementary gene pools for creation of high-performing hybrids. Most likely, breeding efforts based on recurrent reciprocal selection (RRS) will be required to develop heterotic groups, as it has been the case for maize [50]. For identification and further improvement of potential heterotic groups,



molecular markers applied to characterize genetic diversity within elite populations will play a crucial role. Different marker technologies were consistently able to discriminate heterotic groups in maize [51]. It is in addition possible to use markers to identify genetically divergent populations. In ryegrass, various marker technologies including diversity arrays technology (DArT), simple sequence repeats (SSR), and single nucleotide polymorphisms (SNP) have been used to study genetic diversity [52-54]. When studying genetic variation within and among various ryegrass varieties and accessions, most of the genetic variation was found within rather than between ryegrass varieties or accessions [55] and among population variation only explained around 29% of the genetic variation [56]. Analyses of molecular variance (AMOVA) showed that Northern and Southern German ecotypes of ryegrass can be classified as distinct germplasm pools[56]. A more recent comprehensive analysis of population structure in European ryegrass varieties identified two groups representing maritime and continental varieties, respectively [57]. Both studies show that geographically distinct groups of accessions might form different gene pools and, therefore, represent an excellent starting point for RRS programs to establish continuously improved heterotic pools.

Martinez-Reyna and Vogel (2008) assessed different accessions of switchgrass for potential heterotic patterns based on geographic separation. Tetraploid upland and lowland cultivars as well as western and eastern populations and combinations of separate subspecies of switchgrass were evaluated for combining ability. In a spaced plant study, the tetraploid lowland cultivar “Kanlow” and upland cultivar “Summer” represented two distinct heterotic pools [11]. Hultquist, S et al. (1996) used restriction fragment length polymorphisms (RFLP) to discriminate upland and lowland ecotypes, which have demonstrated to be potential heterotic groups. Thus,

geographically separated populations are a starting point to assess potential heterotic groups and this can be further aided by the use of new marker technologies

### ***Biological mechanisms for effective hybrid seed production***

The production of hybrid seed requires a directional cross between a pollen donor and a pollen recipient. In current production schemes, self-pollination of the recipient line or genotype has to be prevented. This can be achieved either by manual emasculation or genetic mechanisms, among which SI and cytoplasmic MS are the most important non-transgenic systems. In perennial grasses like ryegrass and switchgrass with small hermaphrodite flowers, manual emasculation to control pollination is laborious and time consuming in terms of commercial seed production. Therefore, use of SI or MS is crucial for effective hybrid breeding.

SI is a genetic mechanism that allows flowering plants to prevent self-fertilization, thereby maintaining a high degree of heterozygosity (de Nettancourt 1977). The gametophytic SI mechanism in grasses is controlled by a two loci, S and Z (Lundqvist 1962). Involvement of additional loci independent of S and Z has been revealed by studies on the breakdown of SI in perennial ryegrass [58]. Recently, genes differentially expressed during a SI response have been identified using cDNA-AFLP [59] and suppression subtractive hybridization [60]. Still, none of the SI determinants in grasses has been cloned to date. Approaches to find SI loci in ryegrass included genetic mapping and exploitation of synteny between rye and rice [61,60]. Subsequent fine-scale comparative genetic and physical mapping found a high degree of micro-synteny for the Z locus and suggested that combined synteny and map-based cloning strategies are promising for isolating genes involved in SI response in grasses [62]. As the map-based isolation of SI loci is most advanced in perennial ryegrass, information on genetic mechanisms and markers for

genes controlling SI as well as SI-based hybrid breeding strategies might soon become available and transferrable between perennial grass species [19].

For several reasons, SI is a promising tool for hybrid seed production in perennial grasses. SI can be used for combining any pair of genotypes without the necessity to develop male sterile and maintainer genotypes as in the case of cytoplasmic male sterility (CMS). Moreover, it benefits from the ability to vegetatively propagate perennial grasses to multiply parental components for hybrid seed production. More importantly, SI can either be overcome by self-fertility genes as described for perennial ryegrass [58], or by environmental conditions such as high temperature, modifying the expression of incompatibility genes and finally enabling self-fertility by pseudo-compatibility [63].

For switchgrass, a two-locus SI system has been described [34] as in ryegrass. Switchgrass is a species with reported ploidy series ranging from  $2n = 2x = 18$  to  $2n = 12x = 108$  [64]. During meiosis, polyploids may show preferential pairing of duplicated genomes. Occasionally, chromosomes of unlike genomes pair to form quadrivalents or pairs of bivalents. Initial studies in tetraploid genotypes suggest preferential pairing of chromosomes [65]. Furthermore, a recent genetic mapping experiment confirmed a great amount of preferential pairing for almost all chromosomes [66]. High ploidy levels make it difficult to determine the genetics of SI, among others due to the expected large numbers of alleles per locus. Therefore, it is not clear yet, if there are dominance interactions between alleles at the loci involved in SI, which are absent in diploid gametophytic SI [18]. Even though the SI process is not clearly elucidated in switchgrass, progress in fine mapping of S and Z in ryegrass provides an opportunity to identify SI determinants in various perennial grasses with conserved SI system based on S and Z [19]

MS has been described for ryegrass [67] but not for switchgrass so far [68]. Male sterility can be structural or functional [69]. Structural MS can cause abnormal dehiscence of anthers, preventing pollination, whereas functional MS is caused by interruption in either micro- or megagametogenesis driven by cytoplasmic or nuclear genetic factors [70]. Male sterility can either be genic or cytoplasmic, which affects the appropriate breeding strategy [69]. In perennial ryegrass, there are two reported sources of MS, one was induced by intergeneric crosses to *Festuca pratensis* and the other by interspecific crosses between *L. perenne* and *L. multiflorum* [67], the latter causing CMS. In addition, nuclear restorers were identified for *L. perenne* [71,72]. Recently, molecular studies have shown that the presence of an element named LpCMSi in the mitochondrial genome of ryegrass affects CMS [73]. This information can be used to obtain male sterile lines (commonly called A lines, genotypes or populations) by crossing elite genotypes lacking restorer genes onto genotypes carrying CMS or MS genes. CMS systems are generally species-specific due to a high diversity of mitochondrial genes [74,75]. In addition, male sterility and restoration take place in different stages of pollen development for each plant species [76,77]. Restorer genes from *Oryza sativa*, *Petunia hybrid*, *Raphanus sativus*, and *Sorghum bicolor* are members of the pentatricopeptide-repeat (PPR) protein family and are all involved in decreasing the accumulation of CMS locus transcripts [76,77]. This initially suggested homology between restorer genes, but the protein structure and mechanisms for fertility restoration differ between and within species. In maize, different restorer genes have been cloned and characterized such as *Rf1*, *Rf2*, *Rf3*, and *Rf9* which have a high allelic diversity [78]. In contrast to other restorers described previously, *Rf2* in maize is an aldehyde dehydrogenase [79]. This indicates fertility restoration is not conserved within or across species.

Therefore, it is not likely that genetic information for genes involved in ryegrass (C)MS can be transferred across a broader range of grass species based on synteny.

### **Hybrid breeding strategies for perennial grasses**

The different hybrid breeding strategies applicable in perennial grasses are varying in the levels of inbreeding and genetic heterogeneity of hybrid seed, as well as the biological mechanism to be used for hybrid seed production.

#### ***Population hybrids***

Crosses between pairs of populations which are normally a set of subpopulations or cultivars have been used for the development of population hybrids in switchgrass [11]. As a result, progeny will be a mixture of inter- and intrapopulation crosses. With the goal to maximize the amount of hybrid seed (derived from interpopulation crosses), different strategies can be applied: populations can be grown side by side but at different ratios. As a consequence, one population will contribute more pollen to the pollen cloud compared to the other, which can be used as pollen recipient population for seed production. For example, two populations can be grown in a 3:1 ratio of pollen donor to pollen recipient (Figure 1A). Therefore, the seed harvested from the pollen recipient would consist of at least 75% hybrid seed. Moreover, populations used as pollen donors could be improved by conventional breeding schemes for high quantities of pollen production and grown side by side, with the difference that one population will contribute for example three times more pollen to the pollen cloud compared to the recipient population (Figure 1B).

### *Chance hybrids*

The percentage of hybrid seed can be increased if three or more populations are used for intercrossing, as suggested by Brummer (1999). If more populations are added, the amount of non-hybrid seed will decrease even more. The concept of producing a mixture of inter- and intrapopulation hybrids resulting from a cross between two populations has been described as chance hybrids [80], also known as semihybrids [81]. In contrast to population hybrids, a basic semihybrid design as the one proposed by Brummer et al. (1999), requires the identification of at least two populations with good combining ability. In addition, use of at least Syn2 seed within each population would increase the frequency of desired alleles and the inbreeding coefficient within each population which will increase the chance of obtaining hybrid seed. However, as the number of heterotic combinations between groups is limited, the use of more than a pair of populations will result in little or no genetic gain.

The chances of capturing heterosis in a semihybrid design are greater to what is currently obtained with synthetic breeding for different reasons. For instance, in broad-based synthetics, selection causes increase of frequencies desired alleles from Syn1 to Syn3 [81], resulting in an increased inbreeding coefficient. In contrast, in a semihybrid breeding scheme, different alleles will be enriched in the two parental populations prior to hybrid seed production, thereby maximizing the chances to capture heterosis. Alternatively, F1 hybrids can be advanced for hybrid seed production resulting in a similar or higher heterozygosity level as the one described above. (Charles Brummer Personal Communication). In this case SI can be used to avoid self-pollination of plants in hybrid seed production fields

In polyploid switchgrass, preferential pairing reduces the number of possible gametes. This increases the chance to capture heterosis, if genotypes with different allele frequencies are crossed.

The challenge is to select true hybrid progeny in a semihybrid design. Herbicide resistance has been suggested as a possibility to select hybrid progeny in a semihybrid [81]. To have an efficient selection method, the two populations will have to differ in their ability to resist to two different herbicides. The source of resistance can occur naturally in a population or can be introduced via genetic transformation. The progeny of the cross between these two populations need to be sprayed with both herbicides. As a result, most of the surviving progeny should be hybrids between two populations [81]. However, it may be difficult to keep herbicide tolerances restricted to particular parental populations. Moreover, fixation of respective herbicide tolerance genes might be challenging in polyploid species, which leads to a substantial fraction of susceptible hybrids in hybrid seed production.

An alternative strategy is to limit the number of S-Z haplotypes within each of the parent populations (Syn2 population or population hybrids). Thereby, the amount of intrapopulation offspring can be significantly decreased (Fig. 2) and the amount of hybrid seed increased. This strategy will require molecular markers for S and Z. Linked markers are already available for S and Z in ryegrass [48,59,82,62], which can be used to predict S and Z haplotypes. In the near future, functional markers for S and Z may become available, which would increase accuracy of SI haplotype prediction, in particular because. Since S and Z are not linked loci, the possible number of haplotype combinations is large. Selection of a low number of S and Z haplotypes would decrease the chance of intrapopulation crosses. SI underlies frequency dependent selection (FDS), i.e., rare alleles are at a selective advantage. Thus, the number of initial SI haplotypes in a

semi-hybrid design could be rather low and FDS would still ensure sufficient inter-population compatibility and fertility.

S and Z markers could be used in breeding programs to select for unique S-Z haplotypes within a population and different between populations. In a practical example, a single pair cross between two diploid genotypes completely heterozygous for S and Z would result in progeny with sixteen possible genotypes. Around 1092 individuals need to be screened to obtain at least 50 individuals with a unique S-Z genotype. The same has to be done in the other heterotic group, thus approximately 4400 data points are needed to identify parents for each population that accomplish two basic requirements: have genotypes with identical S-Z haplotypes within populations but different S-Z haplotypes among populations. It is noteworthy that the effectiveness of this design relies on the possibility of keeping populations with good combining ability apart and to increase the number of favorable alleles only within groups. With such a scheme, testing thousands of plants for S and Z markers would be a time efficient and non-expensive procedure.

### ***Single cross hybrids***

In a strict sense, the parents of a single cross hybrid are two inbred lines. However, inbred line development in perennial grasses is impaired due to SI and inbreeding depression. Single cross hybrids from heterozygous parent genotypes are a more likely scenario for perennial grass species, and result in segregating F1 populations, comparable to double cross hybrids in maize.

The use of inbred lines will require initial inbreeding and an additional step to restore self-incompatibility. Moreover, inbred line based hybrids are genetically homogenous. This is in contrast to the situation where heterozygous parents are used and the resulting hybrid seed is



heterogeneous (Fig. 3). If S-Z haplotypes are different between hybrid parents, exclusively hybrid seed will be produced. In the short term, obtaining inbred lines from switchgrass may not be feasible due to SI and the fact that SF genes have not been identified and described yet. The fact that heterotic patterns were reported for biomass yield in spaced plant evaluations in switchgrass [11] and ryegrass [56,13] suggest, that it may be possible to identify heterozygous genotypes with superior specific combining ability.

Repeated testing and maintenance of genotypes that display good combining ability is essential for hybrid seed production based on heterozygous parents. Perennial grasses are easy to propagate vegetatively. As a result, selected ryegrass and switchgrass clones can be grown side by side in production fields and a high degree of hybridization will be ensured by SI. Preliminary data show that SI in switchgrass is not fully effective [83] which may require a initial screening of selected genotypes to avoid self-fertilization. Hybrid seed can be harvested from both parents, maximizing seed yield. The main limitation of this approach is the time required to obtain a sufficient number of clones and the time that it will take to establish these clones in production fields. Under greenhouse conditions at Iowa State University, a well-established switchgrass or ryegrass plant can produce up to twelve clones every 6 weeks and it can take up to 5 months before resulting plants can be transplanted to the field. However, in vitro culture techniques can aid to produce large numbers of plantlets in a short period of time. For example, nodal culture of a single switchgrass plant can produce 500 plants ready for planting within 12 weeks [2].

In the longer run, it is preferable to develop inbred lines mainly because seed in large amounts is more desirable and cost effective for establishing hybrid seed production fields compared to generating plantlets by vegetative propagation. This is doable in ryegrass, where self-fertility loci

have been reported [84]. However, there are reports of strong inbreeding depression [85,58]. This poses a challenge for line development in terms of fitness and seed production. Reduced fitness of inbred lines resembles the initial stages of maize hybrid breeding. However, this bottleneck has been overcome by using RRS in maize [50].

Another challenge is production of inbred lines using self-fertility mechanisms, while maintaining the possibility to use SI for controlled crosses in hybrid seed production fields. Self-fertility in perennial ryegrass is monogenically inherited and dominant. Two independent loci affecting SF have been described [84]. Self-fertility loci would be fixed during inbred line development. Therefore, a mechanism to restore SI is required, before controlled crosses can be performed effectively. Up to date, there are no reports of mechanisms to interrupt or break down SF, once SF genes are introduced to a genotype which makes this approach not practical for hybrid seed production. Alternatively, pseudo-compatibility induced by high temperatures can be used to overcome SI temporarily [86,63]. In a study on pseudo-compatibility, seed set increased from an average of 2.3%, obtained under natural conditions, to an average of 31% after temperature treatment [63]. However, control of temperature at a large scale might not be achievable in terms of costs and logistics. Instead, it is possible to use environments with optimal temperatures to achieve pseudo-compatibility for inbred seed production. Still, the risk of using temperature-based mechanisms is, that selfing can be triggered by high temperatures in hybrid seed production fields. Therefore, a mechanism for pseudo-compatibility that can be better manipulated (such as application of a sprayable compound) would be preferable.

In ryegrass, the main limitation is the low amount of seed produced by self-pollination due to a limited number of reproductive tillers. This limitation affects seed yield and also the vegetative propagation coefficient discussed above. Heritability for tiller number is rather low [87].

Therefore, several cycles of selection might be required to obtain inbred lines with sufficient seed yield. In contrast, switchgrass may require a large-scale phenotypic screening of various genotypes or a TILLING population, as a first step towards identification of SF mechanisms as observed in ryegrass.

CMS is an alternative to produce single cross hybrids. The production scheme requires a male sterile A line, and a B line that can be used to maintain male sterility. When using CMS to produce F1 hybrids based on inbred parents, it will be required to introgress self-fertility into elite germplasm or to induce pseudo-compatibility for inbred line production. Male sterility would be introduced to inbred lines within one heterotic group to be pollinated by lines from the other heterotic group. Therefore, this approach is limited to the use of inbred parents because A and B lines have to be isogenic except for their CMS element. In contrast to, e.g., maize CMS breeding schemes [88], restorers are not required since the target trait is biomass yield and not seed production. However, additional efforts are required to maintain nurseries for A and B lines. Another limitation of using CMS in ryegrass is, that it might be leaky under certain environmental conditions, so that viable pollen is produced by “male sterile” genotypes [89], resulting in a certain fraction of self-fertilization [14,15]. Currently, there are no cultivars of ryegrass on the market, developed by using CMS. This is not due to the leaky systems but because the variety candidates developed did not meet the distinctiveness requirements for variety registration (Posselt, personal communication). New sources of CMS have been reported and seem to be more promising for hybrid seed production due to reduced susceptibility to temperatures [74]. In summary, SI or CMS are promising biological mechanisms to enable hybrid seed production in perennial grasses. However, CMS based system can be harvested only in females in contrast to a SI-based system in which hybrid seed can be harvested on both.

Anyway, a major effort to move towards hybrid breeding is to develop heterotic groups to ensure sufficient levels of heterosis and hybrid performance for economic seed production.

Finally it is noteworthy that there are no reports of male sterility in switchgrass. Therefore, using self-incompatibility for hybrid breeding in switchgrass may be the method of choice, as proposed by Martinez-Reyna and Vogel (2008). This scheme might be further improved by using the information available from ryegrass, e.g., sequences for S-Z determinants. This sequence information can be used to identify candidate genes in switchgrass that can be used as markers for parent selection in breeding programs.

## **Conclusions**

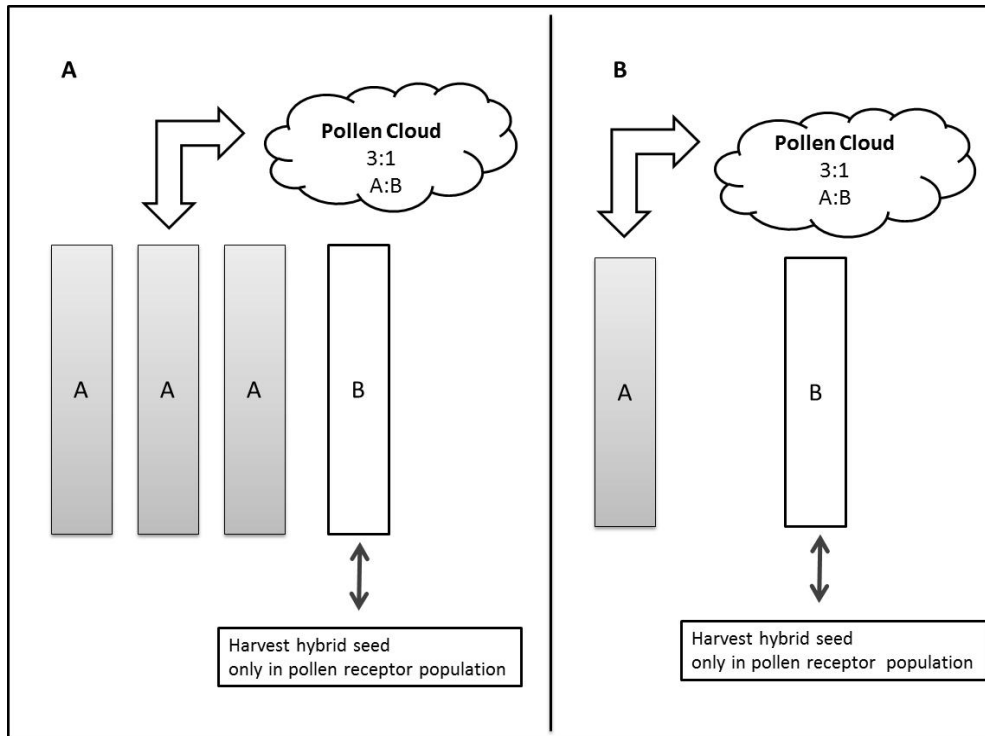
Reportedly up to 30% of increase yield performance has been achieved by traditional breeding [90]. Hybrid breeding may represent an increase in seed production cost, therefore the yield gains obtained by hybrid breeding should be significantly higher than 30%. For instance, Perrin, et al (2008) report a \$16.39 ha<sup>-1</sup> seed cost for a annualized yield of 5.0 Mg ha<sup>-1</sup> [91] , if seed production increases seed cost to \$24.59 ha<sup>-1</sup> (50% increase), hybrid cultivars obtained from any breeding strategy should yield at least 7.5 Mg ha<sup>-1</sup> for farmers to break even. Significant heterosis effects for biomass yield found in initial studies for switchgrass and ryegrass suggest a potential to exploit heterosis to obtain high yielding hybrids To efficiently capture heterosis, efforts to identify and develop heterotic groups exhibiting good combining abilities are required. Molecular markers, and novel genotyping approaches using second generation sequencing will constitute an efficient tool to achieve these goals. Moreover, collaborative efforts of public breeders to identify these genetic patterns will aid to avoid mixing different gene pools. For our model species, both SI and MS are prospective biological mechanisms to control pollination for

efficient hybrid seed production. SI may be the method of choice for population hybrids, if molecular markers allow haplotype identification at S and Z. Allelic markers for SI loci will be very useful for parent selection in population hybrids designs and help establishing a very resource efficient breeding system. CMS is also promising, particularly because no restoration of fertility is required. This is especially important, if inbred lines are used for hybrid seed production. However, it still requires the development of isogenic or nearly isogenic male sterile and maintainer lines, which may increase the time and effort needed to complete a breeding cycle. For species or genotypes, in which SF genes or CMS systems are not available, large-scale phenotypic of various genotypes constitute a first step towards the identification of similar mechanisms in the species of interest. Synteny between grass genomes will allow to use perennial ryegrass as a model for SI and SF, and enable a candidate gene approach to identify orthologues in switchgrass and other bioenergy grasses.

### *Acknowledgements*

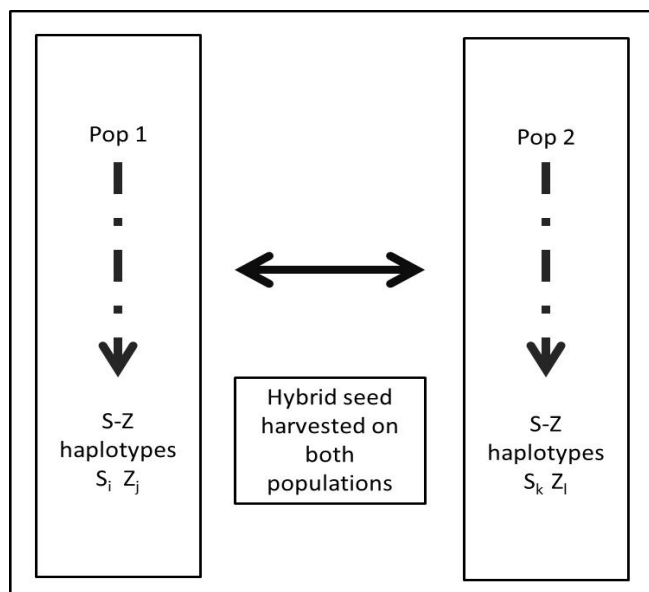
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### **FIGURES AND TABLES**



**Figure 1. Breeding schemes using self-incompatibility to produce population hybrids**

- A) Pollen donor (shaded shapes) and pollen receptor population are grown in a 3:1 ratio**
- B) Pollen donor (A) is a genotype or a set of genotypes that shed more pollen than B genotypes. Therefore A contributes, e.g., three times more pollen to the pollen cloud than B genotypes**



**Figure 2. A breeding scheme using self-incompatibility to produce population hybrid using two populations grown in a 1:1 ratio**

Dashed arrows represent limited compatibility among individuals within the same population and full arrow represents complete pollen-compatibility.  $S_iZ_i$  represents a limited group of haplotypes within the population that differ from the  $S_kZ_k$  set of haplotypes from the other population.

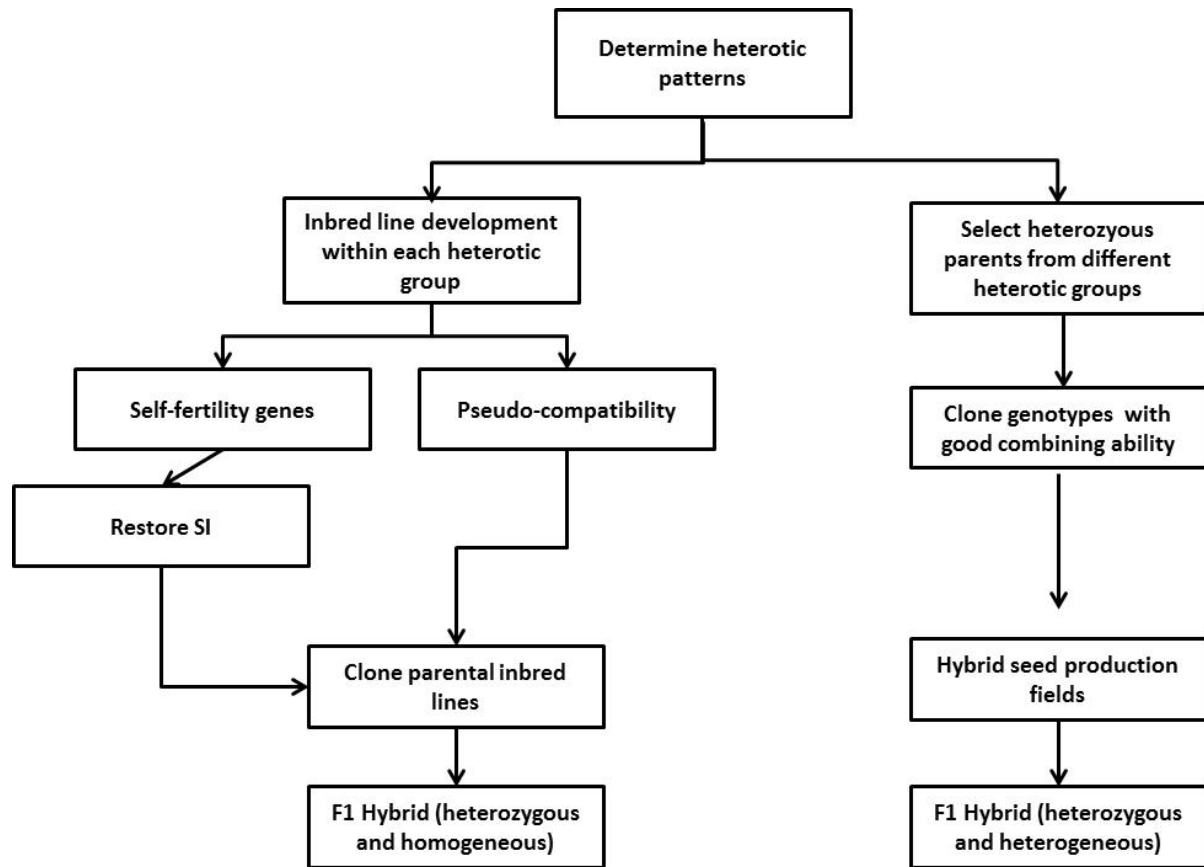


Figure 3. Flow diagram of phases and decision making processes for single cross hybrid production



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